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(54) **Bacillus thuringiensis Cry1Ia-Cry1Ba hybrid toxins**

(57) *Bacillus thuringiensis* hybrid toxin fragment comprising structural domains I, II and III in this order starting from the N-terminal, wherein the domains are derived from at least two different Cry proteins, domain I is domain I of any *Bacillus thuringiensis* Cry protein or a part of said domain or a peptide substantially similar to said domain, domain II is domain II of Cry1Ia or a part of said domain or a peptide substantially similar to said domain, and domain III is domain III of Cry1Ba or a part of said domain or a peptide substantially similar domain.

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Descripti n

1.0. Field of the invention

[0001] The present invention relates to hybrid toxin fragments, and toxins comprising them, derived from *Bacillus thuringiensis* insecticidal crystal proteins.

1.1. Description of the related art

[0002] *Bacillus thuringiensis* (hereinafter B.t.) is a gram-positive bacterium that produces insecticidal crystal proteins during sporulation. The crystal (Cry) proteins form a large (over 160 and growing) family of homologous proteins with unique specificities. Each protein is active against only one or a few insect species. Most reported proteins are active against lepidopterans, with a smaller number showing activity against diptera or coleoptera as reviewed in Schnepf *et al.*, 1998). While initially the Cry proteins were classified according to their activity against one of the abovementioned insect orders (Höfte and Whiteley, 1989), the more recent, now commonly accepted classification is based on amino acid homology (Crickmore *et al.*, 1998).

[0003] The mode of actions of crystal proteins has been partially elucidated. After ingestion by the insect larvae, crystals dissolve in the midgut environment, releasing the proteins as protoxins of 70-140 kDa. The solubilized protoxins are subsequently processed ("activated") by midgut proteases, resulting in a protease-resistant fragment of about 65 kDa, which is the active toxin. The toxin binds to receptors on epithelial cells of the insect midgut and penetrates the membrane. This eventually leads to lysis of the cells and death of the larvae.

[0004] The activity range of a particular delta-endotoxin is to a large extent determined by the occurrence of receptors on the midgut cells of the insect, although solubilization efficiency and proteolytic activition are also factors involved. The importance of binding to receptors is further examplified by the decrease in binding occuring in many instances of resistance to Cry proteins (Ferre *et al.*, 1995).

[0005] Structure determination by X-ray cristallography has shown that three different Cry proteins, and probably all Cry proteins, share a common three domain-structure (Li *et al.*, 1991; Grochulski *et al.*, 1995; Morse *et al.*, 1998). If projected on Cry1 sequences, domain I runs from about amino acid residue 28 to 260, domain II from about 260 to 460 and domain III from about 460 to 620. Since the various toxins have different lengths, the borders of the domains can be defined only approximately. A person skilled in this art will be able to find the borders for the various toxins by comparing the amino acid sequences. The N-terminal domain I consists of 7 α-helices and is considered to be inserting (partially) into the target membrane, and forming part of the pore that eventually kills the insect gut epithelial cells. Both domain II and the C-terminal domain III are very variable, and have been shown to determine activity against specific insects. Although it is not yet clear how these domains individually or acting together may determine specificity, there is strong evidence that both can be involved in binding to (putative) receptors (Lee *et al.*, 1995; Dean *et al.*, 1996; de Maagd *et al.*, 1996b; de Maagd *et al.*, 1999).

[0006] Exchange of domain III between toxins by *in vivo* recombination of their encoding genes may not only alter specificity of a toxin, but can also result in a hybrid toxin with superior toxicity for certain insects (Bosch *et al.*, 1994; de Maagd *et al.*, 1 996a). Intl. Pat. Appl. Publ. No. WO 95/06730 discloses the construction of a hybrid delta-endotoxin consisting of domains I and II of Cry1E, and domain III and protoxin-specific fragment of Cry1C. In bioassays, this hybrid as purified after production in *E. coli* is active against *Manduca sexta, Spodoptera exigua*, and *Mamestra brassicae.* When expressed in and purified from a recombinant *Bacillus thuringiensis* strain, the 1E/1C-hybrid was 1.5 times as active as the most active natural toxin against *S. exigua*, Cry1C. The abovementioned patent application also describes a hybrid delta-endotoxin consisting of domains I and II of Cry1Ab, and domain III of Cry1C. When purified from a recombinant *Bacillus thuringiensis* strain, this Cry1 Ab/Cry1 C-hybrid was approximately 6.6 times as toxic as Cry1C (de Maagd *et al.*, 1 996a). Intl. Pat. Appl. Publ. No. WO 98/22595 discloses a number of hybrid toxins consisting of different combinations of fragments of Cry1Ab, Cry1Ac, Cry1C or Cry1F, of which some have improved acitivity against important pest larvae and/or an extended activity spectrum.

[0007] Producing succesfull hybrids is not easy. From the prior art it appears that only a very limited number of hybrids have an improved activity or a broader host-range specificity. In addition there are many possibilities for recombinantly-engineered crystal proteins, in view of the big number of different crystal proteins (more than 160). Further, the effect of the hybrids produced is not predictable.

2.0 Summary of the invention

[0008] Proteins of the Cry3 (Herrnstadt *et al.*, 1986; McPherson *et al.*, 1988), Cry7 (Lambert *et al.*, 1992) and Cry8 (Sato *et al.*, 1994) classes were found to be active against insects of the order Coleoptera (beetles). Cry3A is the singlemost active protein for the important potato pest Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say).

[0009] It is known that resistance to the endotoxins may occur. Accordingly, resistance to Cry3A may occur in CPB in response to its exposure to Cry3A in transgenic potato. Actually, resistant CPB has already been disclosed. Therefore, it is desirable to have an alternative or replacement for Cry3A.

[0010] The present inventors have found specific hybrid crystal proteins which have a high activity against CPB and therefore can be used as a replacement for Cry3A. The hybrids of the invention comprise domains derived from Cry1Ia and Cry1Ba.

[0011] Cry1 proteins are generally active against lepidopterans (larvae of moths and butterflies). Cry1B and Cry1I have been shown to also have some activity against coleopterans, among which CPB, although their toxicity for CPB is much lower than that of Cry3A (Tailor *et al.*, 1992; Bradley *et al.*, 1995).

[0012] The toxicity of the hybrid crystal proteins of the invention against CPB, as compared to the parental proteins Cry1Ba and Cry1Ia, is surprisingly higher. Additionally a number of the hybrids of the invention have retained the high activity against some Lepidopterans, making them toxins with meaningful dual activity for Coleopterans and Lepidopterans.

[0013] According to the present invention there is provided a B.t. hybrid toxin fragment comprising structural domains I, II and III in this order starting from the N-terminal, wherein the domains are derived from at least two different Cry proteins, domain I is domain I of any of *B. t.* Cry protein or a part of said domain or a peptide substantially similar to said domain , domain II is domain II of Cry1Ia or a part of said domain or a peptide substantially similar to said domain, and domain III is domain III of Cry1Ba or a part of said domain or a peptide substantially similar to said domain. Preferred is a fragment which comprises domain I of Cry1Ia or Cry1Ba or a part of said domain or a peptide substantially similar to said domain, domain II of Cry1Ia or a part of said domain or a peptide substantially similar to said domain, and domain III of Cry1Ba or a part of said domain or a peptide substantially similar to said domain.

[0014] The term " or a peptide substantially similar to said domain" should be understood to mean a peptide having an amino acid sequence which is at least 85% similar to the sequence of the domain. It is preferred that the degree of similarity is at least 90%.

[0015] In the context of the present invention, two amino acid sequences with at least 85% or 90% similarity to each other have at least 85% or 90% identical or conservatively replaced amino acid residues in a like position when aligned optimally allowing for up to 6 gaps with the *proviso* that in respect of the gaps a total not more than 15 amino acid residues are affected. For the purpose of the present invention conservative replacements may be made between amino acids with the following groups:

1. Serine and Threonine;
2. Glutamic acid and Aspartic acid;
3. Arginine and Lysine
4. Asparagine and Glutamine
5. Isoleucine, Leucine, Valine, and Methionine;
6. Phenylalanine, Tyrosine, and Tryptophan
7. Alanine and Glycine

By "or a part of said domain" is meant a peptide comprised by the said domain and having at least 80% of the consecutive sequence thereof.

[0016] It is most particularly preferred that the toxin fragment according to the invention comprises either:

1. An amino acid sequence from about amino acid 20 to about amino acid 641 in SEQ ID NO:2 or
2. An amino acid sequence from about amino acid 20 to about amino acid 632 in SEQ ID NO:4.

[0017] The invention also includes a hybrid toxin comprising the above disclosed fragment or a toxin at least 85% similar to such a hybrid toxin which has substantially similar insecticidal activity.

[0018] The hybrid toxin comprises the three structural domains as defined above and generally may comprise a protoxin segment at the carboxyl end, which is not toxic and is thought to be important for crystal formation. SEQ ID NO: 2 and 4 comprise such pro-toxin segment. Further, the hybrid toxin may comprise a lead sequence, being amino acids 1-19 in SEQ ID NO:2 and 4.

[0019] The invention still further includes pure proteins which are at least 90% identical to the toxin fragments or hybrid toxins according to the invention.

[0020] The invention still further includes recombinant DNA comprising a sequence encoding a protein having an amino acid sequence of the above disclosed toxins or fragments thereof.

[0021] In a preferred embodiment the invention provides recombinant DNA comprising the sequence as shown in SEQ ID NO:1 or 3 or DNA similar thereto encoding a substantially similar protein.

[0022] In a more preferred embodiment the invention provides recombinant DNA comprising the sequence from

about nucleotide 170 to about 1929 in SEQ ID NO:1 or from about nucleotide 147 to about 1896 in SEQ ID NO:3.

[0023] By similar DNA is meant a test sequence which is capable of hybridizing to the inventive recombinant sequence. When the test and inventive sequences are double stranded the nucleic acid constituting the test sequence preferably has a T_m within 20°C of that of the inventive sequence. In the case that the test and inventive sequences are mixed together and denantured simultaneously, The T_m values of the sequences are preferably within 10°C of each other. More preferably the hybridization is performed under stringent conditions, with either the test or inventive DNA preferaby being supported. Thus either a denatured test or inventive sequence is preferably first bound to a support and hybridization is effected for a specified period of time at a temperature of between 50 and 70 °C in double strength citrate buffered saline containing 0.1 % SDS followed by rinsing of the support at the same temperature but with a buffer having a reduced SSC concentration. Depending upon the degree of stringency required, and thus the degree of similarity of the sequences, such reduced concentration buffers are typically single strength SSC containing 0.1 % SDS. Sequences having the greatest degree of similarity are those the hybridization of which is least affected by washing in buffers of reduced concentration. It is most preferred that the test and inventive sequences are so similar that the hybridization between them is substantially unaffected by washing or incubation in one tenth strength sodium citrate buffer containing 0.1% SDS.

[0024] The recombinant DNA may further encode a protein having herbicide resistance, plant growth-promoting, anti-fungal , anti-bacterial, anti-viral and/or anti-nematode properties. In the case that the DNA is to be introduced into a heterologous organism it may be modified to remove known mRNA instability motifs (such as AT rich regions) and polyadenylation signals, and/or codons which are preferred by the organism into which the recombinant DNA is to be inserted may be used so that expression of the thus modified DNA in the said organism yields substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein components of the hybrid toxin or toxin fragments are endogenous.

[0025] The invention still further includes a DNA sequence which is complementary to one which hybridizes under stringent conditions with the recombinant DNA according to the invention.

[0026] Also included in the present invention are: a vector containing such a recombinant (or complementary thereto) DNA sequence; a plant or micro-organism which includes, and enables expression of such DNA; plants transformed with such DNA; the progeny of such plants which contain the DNA stably incorporated and hereditable in a Mendelian manner, an/or the seeds of such plants and such progeny. The invention still further includes protein derived from expression of the said DNA, and insecticidal protein produced by expression of the recombinant DNA within plants transformed therewith.

[0027] The invention still further includes an insecticidal composition containing one or more of the toxin fragments or toxins comprising them according to the invention; a process for combatting insects which comprises exposing them to such fragments or toxins or compositions, and an extraction process for obtaining insecticidal proteins from organic material containing them comprising submitting the material to maceration and solvent extraction.

[0028] The invention will be further apparent from the following description, which describes the production of Bt hybrid toxin fragments according to the invention, taken in conjunction with the associated drawings and sequence listings.

[0029] A person skilled in the art may use different methods to construct the hybrid toxin genes of the invention. Domain encoding regions may be exchanged between two homologous, though different genes by exchanging fragments through restriction enzyme digestion and subsequent ligation, if the same restriction enzyme recognition sites are present at the same position in the two genes. If suitable restriction enzyme recognition sites are not available, new sites can be created at homologous positions in the two genes by various methods of site-directed mutagenesis, as described in the examples below for construction of a common RsrII-site using DNA oligomers SEQ ID: 9 and SEQ ID: 10.

[0030] Alternatively, fragments that are to be combined in a hybrid toxin gene may be produced by PCR-mediated amplification using the original genes as templates, taking care that the ends of the fragments contain compatible restriction enzyme digestion sites. Furthermore, a hybrid toxin encoding DNA fragment may be produced *by in vitro* recombination between overlapping, partially homologous DNA fragments as performed in so-called DNA shuffling experiment (Crameri et al, 1998; Zhao et al., 1998). Also, hybrid toxin encoding DNA fragments may be produced by *in vivo* recombination between two homologous DNA fragments that have been cloned in tandem on a single plasmid (Bosch et al., 1994), followed by screening for the desired recombination events.

3.0 Brief description of the drawings

[0031] **Figure 1** shows the sequence of the relevant part of the *cry1Ba* (top) and *cry1Ia* (bottom) genes, respectively, aligned with the respective oligomers (SEQ ID NO:9 and 10) used for mutagenesis in order to produce a common *Rsr*II restriction enzyme recognition site in both genes. Mutated nucleotides are indicated by asterisks and the newly produced *Rsr*II recognition sites are underlined.

[0032] Figur 2 shows both the *cry1la* gene (solid bar) as well as the *cry1Ba* fragment (open bar) present d as they occur in expression vectors pSN18 and pSN17, respectively. Both genes are inserted in the pKK233-2 derived expression vector for *E. coli,* pBD12. For construction of pSN18 the 3' part of the *cry1Ba* gene, encoding the C-terminal part of the protoxin-specific fragment, was replaced by the corresponding part of the *cry1Ca* gene (base pairs 2038-3627; dashed bar). The positions of the common *Nco*I and *Mun*I restriction sites, as well as of the *Rsr*II sites derived by mutagenesis, and the *BstX*I-site used for exchange of the 3'end of the *cry1Ba* gene are indicated, with nucleotide position numbers below the respective genes.

[0033] Figure 3 shows in a scheme the construction of hybrid gene SN15 from *cry1Ba* (pSN17) and *cry1la* (pSN18), and the subsequent construction of hybrid gene SN19 from *cry1Ba* (pSN17) and pSN15.

[0034] Figure 4 shows the alignment of *cry1Ba* and *cry1la* nucleotide sequences, as present in plasmids pSN17 and pSN18, respectively, around the junctions between the domain II and III (A) and between domain I and II (B). Nucleotide sequences as present in the hybrid genes of SN15 and pSN19 (A) or of pSN19 alone (B) are given in capitals, while identical nucleotides in the DNA that is not present in SN15 and SN19 is represented by dots. The position of the common *Rsr*II and *Mun*I recognition sites are underlined. The site of crossover in the hybrids is represented by the shift of the fully written out nucleotide sequence from top strand to bottom strand in this recognition sites. The amino acid sequence of the resulting hybrid proteins is given in three-letter code below each alignment.

4.0 Brief description of the sequence identifiers

[0035] SEQ ID NO:1 shows the nucleotide sequence of the hybrid protoxin gene SN15.

[0036] SEQ ID NO:2 shows the amino acid sequence of the protein encoded by the gene SN15 shown in SEQ ID NO:1.

[0037] SEQ ID NO:3 shows the nucleotide sequence of the hybrid protoxin gene SN19.

[0038] SEQ ID NO:4 shows the amino acid sequence of the protein encoded by the gene SN19 shown in SEQ ID NO:3.

[0039] SEQ ID NO:5 shows the nucleotide sequence of the modified *cry1Ba* gene as used in expression vector pSN17.

[0040] SEQ ID NO:6 shows the amino acid sequence of the protein encoded by the *cry1Ba* gene shown in SEQ ID NO:5.

[0041] SEQ ID NO:7 shows the nucleotide sequence of the modified *cry1la* gene as used in expression vector pSN18.

[0042] SEQ ID NO:8 shows the amino acid sequence of the protein encoded by the *cry1la* gene shown in SEQ ID NO:7.

[0043] SEQ ID NO:9 shows the nucleotide sequence of the oligomer used for mutagenesis of the *cry1Ba* gene.

[0044] SEQ ID NO:10 shows the nucleotide sequence of the oligomer used for mutagenesis of the *cry1la* gene.

5.0 Examples

5.1 DNA manipulations

[0045] All recombinant DNA techniques are as described by Ausubel et al. (1997). Mutagenesis, restriction enzyme digestion and ligation are performed according the instructions of the manufacturers. DNA sequencing is performed by the dideoxytriphosphate methode with fluorescent dyes attached to the dideoxynucleotides. Analysis is automated by using an Applied Biosystems 370A nucleotide sequence analyzer.

5.2 Expression vectors

[0046] All Cry protein expression vectors are based on pBD12, a derivative of pKK233-2 (Bosch *et al.*, 1994). For expression of Cry3Aa protein, the full *cry3Aa* gene is cloned into pBD12, giving expression plasmid pMH10. For cloning purposes both *cry1Ba* as well as *cry1la* are mutagenized in order to contain a *Nco*I-site overlapping with the start codon. For production of Cry1Ba protein, a *NcoI-BstX*I (bases 1-1977) fragment of *cry1Ca* in pBD150 (Bosch *et al.*, 1994) is replaced by the corresponding fragment of *cry1Ba* (bases 1-2037), resulting in *cry1Ba* expression vector pMH19. pMH19 therefor contains the 5'active toxin encoding part of Cry1Ba and 3' protoxin specific part of Cry1Ca. Cry1la expression vector pBD172 contains the full *cry1la* gene with the *Spe*I-site (base 2180) fused to the SpeI-site in the polylinker of pBluescript SK$^+$.

5.3 Mutagenesis of cry1Ba and cry1la

[0047] In order to be able to directly exchange the domain III encoding regions between *cry1Ba* and *cry1la,* a new

common restriction enzyme recognition site is made in both genes by site directed mutagenesis. Complementary mutagenic oligonucleotide pairs are used to create unique RsrII-sites at positions 1464 and 1488 of *cry1Ba* (pMH19) and *cry1Ia* (pBD172), respectively (see Fig. 1), using the using the QuickChange™ kit (Stratagene). These mutations do not change the encoded proteins. This results in two new expression plasmids, pSN17 (Cry1Ba, Seq. ID NO: 5) and pSN18 (Cry1Ia, Seq. ID NO: 7), respectively (Fig. 2). The unique *Rsr*II restriction sites at the border of the domain II and domain III encoding regions, together with the common *Mun*I-sites at the border between the domain I and domain II encoding regions allowed simple swapping of domain encoding regions between the two genes.

5.4 Construction of hybrid toxins

[0048] Fig. 3 schematically shows the construction of two novel hybrid toxins. Both pSN18 and pSN17 are digested with *Nco*I and *Rsr*II. Subsequently, the 1488 base fragment of pSN18, containing the domain I and II encoding fragment of *cry1Ia* is ligated into the corresponding sites of the pSN17-derived fragment containing the 3'portion of *cry1Ba*. This results in plasmid pSN15 encoding a 1Ia/1Ia/1Ba-hybrid (Seq. ID NO:1). The nucleotide sequence of the cross-over region with the encoded amino acid sequence is shown in Fig. 4A. Subsequently a *Nco*I-*Mun*I (base 1-896) fragment encoding domain I of Cry1Ia from pSN15 is replaced by the corresponding fragment encoding domain I of Cry1Ba, derived from pSN17. This results in 1Ba/1Ia/1 Ba-hybrid encoding plasmid pSN19 (Seq. ID. NO:3). The nucleotide sequence of the crossover region with the encoded amino acid sequence is shown in Fig. 4B.

5.5 Protein isolation and insect bioassays.

[0049] For large-scale production, all parental and hybrid protoxins are expressed in *E. coli* strain XL-1 and extracted as described earlier (Bosch *et al.*, 1994). Solubilized protoxins are dialyzed overnight in 25 mM $NaHC_3$, 100 mM NaCl, pH10. Protein concentrations are estimated by SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis). To test toxicity to Colorado potato beetle (CPB), leaflets of greenhouse grown potato culitivar Desiree are dipped in toxin dilutions in water containing 0.01 % Tween-20. After drying of the leaves to the air they are transferred to petri dishes and 10 neonate CPB larvae are placed on each leaf. After incubation for two days at 28°C, the leaves are replaced by fresh leaves dipped in identical protoxin dilutions. Mortality is scored after 4 days. LC_{50} (concentration with 50% mortality) and 95% fiducial limits are determined by Probit analysis of results from three or more independent experiments, using the PoloPC computer program (Russel *et al.*, 1977).

5.6 Toxicity of wild type and hybrid proteins to Colorado potato beetle

[0050] Table 1 shows the toxicity of the hybrid proteins SN15 and SN19 against Colorado potato beetle (CPB), as compared to the parental proteins Cry1Ba and Cry1Ia, and to the most CPB-active natural toxin available, Cry3A. 1Ia/1Ia/1Ba-hybrid SN15 shows slightly higher toxicity (lower LC50) than its best parental toxin, Cry1Ia, on a per weight basis. When considering that the molecular weight of the SN15 protein is considerably larger than that of Cry1Ia, it follows that SN15 performs even better on a per mol basis. 1Ba/1Ia/1Ba-hybrid protein SN19 is even considerably more toxic than SN15.

Table 1.

Toxicity of wild type and hybrid protoxins to Colorado potato beetles.				
Protoxin	LC_{50}[a]	95% fiducial limits [a]	MW[b]	Relative toxicity[c]
MH10 (Cry3A)	1.8	1.4-2.5	74.0	100
SN17(Cry1Ba)	142	105-198	137.4	1
SN18(Cry1Ia)	34	23-47	81.3	6
SN15	22	14-35	138.0	15
SN19	8	5-11	137.2	42

[a]Concentration in microgram per milliliter of dipping solution. LC_{50}: concentration which leads to 50% mortality;
[b]Approximate molecular weight in kiloDaltons.
[c]Relative toxicity on molar basis in percents, with toxicity of Cry3A set at 100%.

Although the present invention has been particularly described for the production of SN15 and SN19 hybrid toxins, the

skilled scientist will appreciate that other hybrid toxins with improved insecticidal characteristics may be produced according to the invention. Hybrid toxins containing th combination of Cry1Ia domain II and Cry1Ba domain III, with various combinations of domain I and C-terminal extensions may be made. Moreover, the gene encoding SN15, SN19 and/or other hybrids may be transferred into strains of B.t. and/or integrated into the chromosome of strains of B.t., of other bacteria or be introduced into plant genomes to provide for in situ insecticidal activity within the plant per se. In this regard, it is particularly preferred that the recombinant DNA encoding the toxins is modified in that sequences which are detrimental to high level expression in plants are removed and in that codons which are preferred by the plant are used. This should lead to production in the plant of a substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein components of the hybrid toxins or toxin fragments are endogenous.

6.0 References

[0051] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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Pro Glu Ala Thr Gly Gln Pro Asn Tyr Glu Ser Tyr Ser His Arg Leu
450 455 460

Ser His Ile Gly Leu Ile Ser Ala Ser His Val Lys Ala Ser Val Tyr
465 470 475 480

Ser Trp Thr His Arg Ser Ala Asp Arg Thr Asn Thr Ile Gly Pro Asn
485 490 495

Arg Ile Thr Gln Ile Pro Met Val Lys Ala Ser Glu Leu Pro Gln Gly
500 505 510

Thr Thr Val Val Arg Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Arg
515 520 525

Arg Thr Asn Thr Gly Gly Phe Gly Pro Ile Arg Val Thr Val Asn Gly
530 535 540

Pro Leu Thr Gln Arg Tyr Arg Ile Gly Phe Arg Tyr Ala Ser Thr Val
545 550 555 560

Asp Phe Asp Phe Phe Val Ser Arg Gly Gly Thr Thr Val Asn Asn Phe
565 570 575

Arg Phe Leu Arg Thr Met Asn Ser Gly Asp Glu Leu Lys Tyr Gly Asn
580 585 590

Phe Val Arg Arg Ala Phe Thr Thr Pro Phe Thr Phe Thr Gln Ile Gln
595 600 605

Asp Ile Ile Arg Thr Ser Ile Gln Gly Leu Ser Gly Asn Gly Glu Val
610 615 620

Tyr Ile Asp Lys Ile Glu Ile Ile Pro Val Thr Ala Thr Phe Glu Ala
625 630 635 640

Glu Tyr Asp Leu Glu Arg Ala Gln Glu Ala Val Asn Ala Leu Phe Thr
645 650 655

Asn Thr Asn Pro Arg Arg Leu Lys Thr Asp Val Thr Asp Tyr His Ile
660 665 670

Asp Gln Val Ser Asn Leu Val Asp Cys Leu Ser Asp Glu Phe Cys Leu
675 680 685

Asp Glu Lys Arg Glu Leu Ser Glu Lys Val Lys His Ala Lys Arg Leu
690 695 700

Ser Asp Glu Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg Gly Ile Asn
705 710 715 720

Arg Gln Pro Asp Arg Gly Trp Arg Gly Ser Thr Asp Ile Thr Ile Gln
725 730 735

Gly Gly Asp Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro Gly Thr
740 745 750

Val Asp Glu Cys Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser
755 760 765

Lys Leu Lys Ala Tyr Thr Arg Tyr Glu Leu Arg Gly Tyr Ile Glu Asp
770 775 780

Ser Gln Asp Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala Lys His Glu
785 790 795 800

Ile Val Asn Val Pro Gly Thr Gly Ser Leu Trp Pro Leu Ser Ala Gln
805 810 815

Ser Pro Ile Gly Lys Cys Gly Glu Pro Asn Arg Cys Ala Pro His Leu
820 825 830

Glu Trp Asn Pro Asp Leu Asp Cys Ser Cys Arg Asp Gly Glu Lys Cys
835 840 845

Ala His His Ser His His Phe Thr Leu Asp Ile Asp Val Gly Cys Thr
850 855 860

```
Asp Leu Asn Glu Asp Leu Gly Val Trp Val Ile Phe Lys Ile Lys Thr
865                 870                 875                 880

Gln Asp Gly His Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu Glu Lys
                885                 890                 895

Pro Leu Leu Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu Lys Lys
            900                 905                 910

Trp Arg Asp Lys Arg Glu Lys Leu Gln Leu Glu Thr Asn Ile Val Tyr
        915                 920                 925

Lys Glu Ala Lys Glu Ser Val Asp Ala Leu Phe Val Asn Ser Gln Tyr
    930                 935                 940

Asp Arg Leu Gln Val Asp Thr Asn Ile Ala Met Ile His Ala Ala Asp
945                 950                 955                 960

Lys Arg Val His Arg Ile Arg Glu Ala Tyr Leu Pro Glu Leu Ser Val
                965                 970                 975

Ile Pro Gly Val Asn Ala Ala Ile Phe Glu Glu Leu Glu Gly Arg Ile
            980                 985                 990

Phe Thr Ala Tyr Ser Leu Tyr Asp Ala Arg Asn Val Ile Lys Asn Gly
        995                 1000                1005

Asp Phe Asn Asn Gly Leu Leu Cys Trp Asn Val Lys Gly His Val Asp
    1010                1015                1020

Val Glu Glu Gln Asn Asn His Arg Ser Val Leu Val Ile Pro Glu Trp
1025                1030                1035                1040

Glu Ala Glu Val Ser Gln Glu Val Arg Val Cys Pro Gly Arg Gly Tyr
                1045                1050                1055

Ile Leu Arg Val Thr Ala Tyr Lys Glu Gly Tyr Gly Glu Gly Cys Val
            1060                1065                1070

Thr Ile His Glu Ile Glu Asp Asn Thr Asp Glu Leu Lys Phe Ser Asn
        1075                1080                1085

Cys Val Glu Glu Glu Val Tyr Pro Asn Asn Thr Val Thr Cys Asn Asn
    1090                1095                1100

Tyr Thr Gly Thr Gln Glu Glu Tyr Glu Gly Thr Tyr Thr Ser Arg Asn
1105                1110                1115                1120
```

```
Gln Gly Tyr Asp Glu Ala Tyr Gly Asn Asn Pro Ser Val Pro Ala Asp
            1125                1130                1135

Tyr Ala Ser Val Tyr Glu Glu Lys Ser Tyr Thr Asp Gly Arg Arg Glu
        1140                1145                1150

Asn Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp Tyr Thr Pro Leu Pro
    1155                1160                1165

Ala Gly Tyr Val Thr Lys Asp Leu Glu Tyr Phe Pro Glu Thr Asp Lys
1170                1175                1180

Val Trp Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe Ile Val Asp Ser
1185                1190                1195                1200

Val Glu Leu Leu Leu Met Glu Glu
                1205
```

<210> 5
<211> 3627
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: modified
crylBa gene

<400> 5

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caaatggatc tattaccaga tgctcgtatt gaggatagct tgtgtatagc cgaggggaac 120
aatatcgatc catttgttag cgcatcaaca gtccaaacgg gtattaacat agctggtaga 180
atactaggcg tattgggcgt accgtttgct ggacaactag ctagttttta tagttttctt 240
gttggtgaat tatggccccg cggcagagat cagtgggaaa ttttcctaga acatgtcgaa 300
caacttataa atcaacaaat aacagaaaat gctaggaata cggctcttgc tcgattacaa 360
ggtttaggag attccttcag agcctatcaa cagtcacttg aagattggct agaaaaccgt 420
gatgatgcaa gaacgagaag tgttctttat acccaatata tagctttaga acttgatttt 480
cttaatgcga tgccgctttt cgcaattaga aaccaagaag ttccattatt gatggtatat 540
gctcaagctg caaatttaca cctattatta ttgagagatg cctctctttt tggtagtgaa 600
tttgggctta catcgcagga aattcaacgc tattatgagc gccaagtgga acgaacgaga 660
gattattccg actattgcgt agaatggtat aatacaggtc taaatagctt gagagggaca 720
aatgccgcaa gttgggtacg gtataatcaa ttccgtagag atctaacgtt aggagtatta 780
gatctagtgg cactattccc aagctatgac actcgcactt atccaataaa tacgagtgct 840
cagttaacaa gagaagttta tacagacgca attggagcaa caggggtaaa tatggcaagt 900
atgaattggt ataataataa tgcaccttcg ttctctgcca tagaggctgc ggctatccga 960
agcccgcatc tacttgattt tctagaacaa cttacaattt ttagcgcttc atcacgatgg 1020
agtaatacta ggcatatgac ttattggcgg gggcacacga ttcaatctcg gccaatagga 1080
```

```
ggcggattaa atacctcaac gcatggggct accaatactt ctattaatcc tgtaacatta 1140
cggttcgcat ctcgagacgt ttataggact gaatcatatg caggagtgct tctatgggga 1200
atttaccttg aacctattca tggtgtccct actgttaggt ttaattttac gaaccctcag 1260
aatatttctg atagaggtac cgctaactat agtcaacctt atgagtcacc tgggcttcaa 1320
ttaaaagatt cagaaactga attaccacca gaaacaacag aacgaccaaa ttatgaatct 1380
tacagtcaca ggttatctca tataggtata attttacaat ccagggtgaa tgtaccggta 1440
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gtaactgtta acggaccatt aacacaaaga tatcgtatag gattccgcta tgcttcaact 1680
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gcagaatatg atttagaaag agcgcaagag gcggtgaatg ctctgtttac taatacgaat 1980
ccaagaagat tgaaaacaga tgtgacagat tatcatattg atcaagtatc caatttagtg 2040
gattgtttat cagatgaatt ttgtctggat gaaaagcgag aattgtccga gaaagtcaaa 2100
catgcgaagc gactcagtga tgagcggaat ttacttcaag atccaaactt cagagggatc 2160
aatagacaac cagaccgtgg ctggagagga agtacagata ttaccatcca aggaggagat 2220
gacgtattca aagagaatta cgtcacacta ccgggtaccg ttgatgagtg ctatccaacg 2280
tatttatatc agaaaataga tgagtcgaaa ttaaaagctt atacccgtta tgaattaaga 2340
gggtatatcg aagatagtca agacttagaa atctatttga tccgttacaa tgcaaaacac 2400
gaaatagtaa atgtgccagg cacgggttcc ttatggccgc tttcagccca aagtccaatc 2460
ggaaagtgtg gagaaccgaa tcgatgcgcg ccacaccttg aatggaatcc tgatctagat 2520
tgttcctgca gagacgggga aaaatgtgca catcattccc atcatttcac cttggatatt 2580
gatgttggat gtacagactt aaatgaggac ttaggtgtat gggtgatatt caagattaag 2640
acgcaagatg gccatgcaag actagggaat ctagagtttc tcgaagagaa accattatta 2700
ggggaagcac tagctcgtgt gaaaagagcg gagaagaagt ggagagacaa acgagagaaa 2760
ctgcagttgg aaacaaatat tgtttataaa gaggcaaaag aatctgtaga tgctttattt 2820
gtaaactctc aatatgatag attacaagtg gatacgaaca tcgcgatgat tcatgcggca 2880
gataaacgcg ttcatagaat ccgggaagcg tatctgccag agttgtctgt gattccaggt 2940
gtcaatgcgg ccatttttcga agaattagag ggacgtattt ttacagcgta ttccttatat 3000
gatgcgagaa atgtcattaa aaatggcgat ttcaataatg gcttattatg ctggaacgtg 3060
aaaggtcatg tagatgtaga agagcaaaac aaccaccgtt cggtccttgt tatcccagaa 3120
tgggaggcag aagtgtcaca agaggttcgt gtctgtccag gtcgtggcta tatccttcgt 3180
gtcacagcat ataaagaggg atatggagag ggctgcgtaa cgatccatga gatcgaagac 3240
aatacagacg aactgaaatt cagcaactgt gtagaagagg aagtatatcc aaacaacaca 3300
gtaacgtgta ataattatac tgggactcaa gaagaatatg agggtacgta cacttctcgt 3360
aatcaaggat atgacgaagc ctatggtaat aaccccttccg taccagctga ttacgcttca 3420
gtctatgaag aaaaatcgta tacagatgga cgaagagaga atccttgtga atctaacaga 3480
ggctatgggg attacacacc actaccggct ggttatgtaa caaaggattt agagtacttc 3540
ccagagaccg ataaggtatg gattgagatc ggagaaacag aaggaacatt catcgtggat 3600
agcgtggaat tactccttat ggaggaa                                      3627
```

<210> 6
<211> 1209
<212> PRT

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: protein encoded by the modified cry1Ba gene

<400> 6

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Met Thr Ser Asn Arg Lys Asn Glu Asn Glu Ile Ile Asn Ala Val Ser
  1               5                  10                  15

Asn His Ser Ala Gln Met Asp Leu Leu Pro Asp Ala Arg Ile Glu Asp
             20                  25                  30

Ser Leu Cys Ile Ala Glu Gly Asn Asn Ile Asp Pro Phe Val Ser Ala
         35                  40                  45

Ser Thr Val Gln Thr Gly Ile Asn Ile Ala Gly Arg Ile Leu Gly Val
     50                  55                  60

Leu Gly Val Pro Phe Ala Gly Gln Leu Ala Ser Phe Tyr Ser Phe Leu
 65                  70                  75                  80

Val Gly Glu Leu Trp Pro Arg Gly Arg Asp Gln Trp Glu Ile Phe Leu
                 85                  90                  95

Glu His Val Glu Gln Leu Ile Asn Gln Gln Ile Thr Glu Asn Ala Arg
            100                 105                 110

Asn Thr Ala Leu Ala Arg Leu Gln Gly Leu Gly Asp Ser Phe Arg Ala
        115                 120                 125

Tyr Gln Gln Ser Leu Glu Asp Trp Leu Glu Asn Arg Asp Asp Ala Arg
    130                 135                 140

Thr Arg Ser Val Leu Tyr Thr Gln Tyr Ile Ala Leu Glu Leu Asp Phe
145                 150                 155                 160

Leu Asn Ala Met Pro Leu Phe Ala Ile Arg Asn Gln Glu Val Pro Leu
                165                 170                 175

Leu Met Val Tyr Ala Gln Ala Ala Asn Leu His Leu Leu Leu Leu Arg
            180                 185                 190

Asp Ala Ser Leu Phe Gly Ser Glu Phe Gly Leu Thr Ser Gln Glu Ile
        195                 200                 205

Gln Arg Tyr Tyr Glu Arg Gln Val Glu Arg Thr Arg Asp Tyr Ser Asp
    210                 215                 220
```

Tyr Cys Val Glu Trp Tyr Asn Thr Gly Leu Asn Ser Leu Arg Gly Thr
225 230 235 240

Asn Ala Ala Ser Trp Val Arg Tyr Asn Gln Phe Arg Arg Asp Leu Thr
245 250 255

Leu Gly Val Leu Asp Leu Val Ala Leu Phe Pro Ser Tyr Asp Thr Arg
260 265 270

Thr Tyr Pro Ile Asn Thr Ser Ala Gln Leu Thr Arg Glu Val Tyr Thr
275 280 285

Asp Ala Ile Gly Ala Thr Gly Val Asn Met Ala Ser Met Asn Trp Tyr
290 295 300

Asn Asn Asn Ala Pro Ser Phe Ser Ala Ile Glu Ala Ala Ala Ile Arg
305 310 315 320

Ser Pro His Leu Leu Asp Phe Leu Glu Gln Leu Thr Ile Phe Ser Ala
325 330 335

Ser Ser Arg Trp Ser Asn Thr Arg His Met Thr Tyr Trp Arg Gly His
340 345 350

Thr Ile Gln Ser Arg Pro Ile Gly Gly Gly Leu Asn Thr Ser Thr His
355 360 365

Gly Ala Thr Asn Thr Ser Ile Asn Pro Val Thr Leu Arg Phe Ala Ser
370 375 380

Arg Asp Val Tyr Arg Thr Glu Ser Tyr Ala Gly Val Leu Leu Trp Gly
385 390 395 400

Ile Tyr Leu Glu Pro Ile His Gly Val Pro Thr Val Arg Phe Asn Phe
405 410 415

Thr Asn Pro Gln Asn Ile Ser Asp Arg Gly Thr Ala Asn Tyr Ser Gln
420 425 430

Pro Tyr Glu Ser Pro Gly Leu Gln Leu Lys Asp Ser Glu Thr Glu Leu
435 440 445

Pro Pro Glu Thr Thr Glu Arg Pro Asn Tyr Glu Ser Tyr Ser His Arg
450 455 460

Leu Ser His Ile Gly Ile Ile Leu Gln Ser Arg Val Asn Val Pro Val
465 470 475 480

Tyr Ser Trp Thr His Arg Ser Ala Asp Arg Thr Asn Thr Ile Gly Pro
485 490 495

Asn Arg Ile Thr Gln Ile Pro Met Val Lys Ala Ser Glu Leu Pro Gln
500 505 510

Gly Thr Thr Val Val Arg Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu
515 520 525

Arg Arg Thr Asn Thr Gly Gly Phe Gly Pro Ile Arg Val Thr Val Asn
530 535 540

Gly Pro Leu Thr Gln Arg Tyr Arg Ile Gly Phe Arg Tyr Ala Ser Thr
545 550 555 560

Val Asp Phe Asp Phe Phe Val Ser Arg Gly Gly Thr Thr Val Asn Asn
565 570 575

Phe Arg Phe Leu Arg Thr Met Asn Ser Gly Asp Glu Leu Lys Tyr Gly
580 585 590

Asn Phe Val Arg Arg Ala Phe Thr Thr Pro Phe Thr Phe Thr Gln Ile
595 600 605

Gln Asp Ile Ile Arg Thr Ser Ile Gln Gly Leu Ser Gly Asn Gly Glu
610 615 620

Val Tyr Ile Asp Lys Ile Glu Ile Ile Pro Val Thr Ala Thr Phe Glu
625 630 635 640

Ala Glu Tyr Asp Leu Glu Arg Ala Gln Glu Ala Val Asn Ala Leu Phe
645 650 655

Thr Asn Thr Asn Pro Arg Arg Leu Lys Thr Asp Val Thr Asp Tyr His
660 665 670

Ile Asp Gln Val Ser Asn Leu Val Asp Cys Leu Ser Asp Glu Phe Cys
675 680 685

Leu Asp Glu Lys Arg Glu Leu Ser Glu Lys Val Lys His Ala Lys Arg
690 695 700

Leu Ser Asp Glu Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg Gly Ile
705 710 715 720

Asn Arg Gln Pro Asp Arg Gly Trp Arg Gly Ser Thr Asp Ile Thr Ile
725 730 735

Gln Gly Gly Asp Asp Val Phe Lys Glu Asn Tyr Val Thr Leu Pro Gly
740 745 750

Thr Val Asp Glu Cys Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu
755 760 765

Ser Lys Leu Lys Ala Tyr Thr Arg Tyr Glu Leu Arg Gly Tyr Ile Glu
770 775 780

Asp Ser Gln Asp Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala Lys His
785 790 795 800

Glu Ile Val Asn Val Pro Gly Thr Gly Ser Leu Trp Pro Leu Ser Ala
805 810 815

Gln Ser Pro Ile Gly Lys Cys Gly Glu Pro Asn Arg Cys Ala Pro His
820 825 830

Leu Glu Trp Asn Pro Asp Leu Asp Cys Ser Cys Arg Asp Gly Glu Lys
835 840 845

Cys Ala His His Ser His His Phe Thr Leu Asp Ile Asp Val Gly Cys
850 855 860

Thr Asp Leu Asn Glu Asp Leu Gly Val Trp Val Ile Phe Lys Ile Lys
865 870 875 880

Thr Gln Asp Gly His Ala Arg Leu Gly Asn Leu Glu Phe Leu Glu Glu
885 890 895

Lys Pro Leu Leu Gly Glu Ala Leu Ala Arg Val Lys Arg Ala Glu Lys
900 905 910

Lys Trp Arg Asp Lys Arg Glu Lys Leu Gln Leu Glu Thr Asn Ile Val
915 920 925

Tyr Lys Glu Ala Lys Glu Ser Val Asp Ala Leu Phe Val Asn Ser Gln
930 935 940

Tyr Asp Arg Leu Gln Val Asp Thr Asn Ile Ala Met Ile His Ala Ala
945 950 955 960

Asp Lys Arg Val His Arg Ile Arg Glu Ala Tyr Leu Pro Glu Leu Ser
965 970 975

Val Ile Pro Gly Val Asn Ala Ala Ile Phe Glu Glu Leu Glu Gly Arg
980 985 990

```
Ile Phe Thr Ala Tyr Ser Leu Tyr Asp Ala Arg Asn Val Ile Lys Asn
        995                 1000                1005

Gly Asp Phe Asn Asn Gly Leu Leu Cys Trp Asn Val Lys Gly His Val
   1010                1015                1020

Asp Val Glu Glu Gln Asn Asn His Arg Ser Val Leu Val Ile Pro Glu
1025                1030                1035                1040

Trp Glu Ala Glu Val Ser Gln Glu Val Arg Val Cys Pro Gly Arg Gly
                1045                1050                1055

Tyr Ile Leu Arg Val Thr Ala Tyr Lys Glu Gly Tyr Gly Glu Gly Cys
            1060                1065                1070

Val Thr Ile His Glu Ile Glu Asp Asn Thr Asp Glu Leu Lys Phe Ser
        1075                1080                1085

Asn Cys Val Glu Glu Glu Val Tyr Pro Asn Asn Thr Val Thr Cys Asn
   1090                1095                1100

Asn Tyr Thr Gly Thr Gln Glu Glu Tyr Glu Gly Thr Tyr Thr Ser Arg
1105                1110                1115                1120

Asn Gln Gly Tyr Asp Glu Ala Tyr Gly Asn Asn Pro Ser Val Pro Ala
                1125                1130                1135

Asp Tyr Ala Ser Val Tyr Glu Glu Lys Ser Tyr Thr Asp Gly Arg Arg
            1140                1145                1150

Glu Asn Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp Tyr Thr Pro Leu
        1155                1160                1165

Pro Ala Gly Tyr Val Thr Lys Asp Leu Glu Tyr Phe Pro Glu Thr Asp
   1170                1175                1180

Lys Val Trp Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe Ile Val Asp
1185                1190                1195                1200

Ser Val Glu Leu Leu Leu Met Glu Glu
                1205
```

<210> 7
<211> 2160
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: modified cry1Ia gene

<400> 7
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atctctacgg attcactaaa aaatgaaaca gatatagaat tacaaaacat taatcatgaa 120
gattgtttga aaatgtctga gtatgaaaat gtagagccgt ttgttagtgc atcaacaatt 180
caaacaggta ttggtattgc gggtaaaata cttggtaccc taggcgttcc ttttgcagga 240
caagtagcta gtctttatag ttttatctta ggtgagctat ggcctaaggg gaaaaatcaa 300
tgggaaatct ttatggaaca tgtagaagag attattaatc aaaaaatatc aacttatgca 360
agaaataaag cacttacaga cttgaaagga ttaggagatg ccttagctgt ctaccatgat 420
tcgcttgaaa gttgggttgg aaatcgtaat aacacaaggg ctaggagtgt tgtcaagagc 480
caatatatcg cattagaatt gatgttcgtt cagaaactac cttcttttgc agtgtctgga 540
gaggaggtac cattattacc gatatatgcc caagctgcaa atttacattt gttgctatta 600
agagatgcat ctatttttgg aaaagagtgg ggattatcat cttcagaaat ttcaacattt 660
tataaccgtc aagtcgaacg agcaggagat tattcctacc attgtgtgaa atggtatagc 720
acaggtctaa ataacttgag gggtacaaat gccgaaagtt gggtacgata taatcaattc 780
cgtagagaca tgactttaat ggtactagat ttagtggcac tatttccaag ctatgataca 840
caaatgtatc caattaaaac tacagcccaa cttacaagag aagtatatac agacgcaatt 900
gggacagtac atccgcatcc aagttttaca agtacgactt ggtataataa taatgcacct 960
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caagttacaa tttacagctt attaagtcga tggagtaaca ctcagtatat gaatatgtgg 1080
ggaggacata aactagaatt ccgaacaata ggaggaacgt taaatatctc aacacaagga 1140
tctactaata cttctattaa tcctgtaaca ttaccgttca cttctcgaga cgtctatagg 1200
actgaatcat tggcagggct gaatctattt ttaactcaac ctgttaatgg agtacctagg 1260
gttgattttc attggaaatt cgtcacacat ccgatcgcat ctgataattt ctattatcca 1320
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acaattgagc caaatagcat tacacaaata ccattagtaa aagctttcaa tctgtcttca 1560
ggtgccgctg tagtgagagg accaggattt acaggtgggg atatccttcg aagaacgaat 1620
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gctattaatc aaggtaattt ttcagcaact atgaatagag gagaggactt agactataaa 1800
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acaataggtg cttggaactt ctcttcaggt aacgaagttt atatagatag aattgaattt 1920
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<210> 8
<211> 719
<212> PRT

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: protein encoded by the modified cryIIa gene

<400> 8

```
Met Lys Leu Lys Asn Gln Asp Lys His Gln Ser Phe Ser Ser Asn Ala
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Lys Val Asp Lys Ile Ser Thr Asp Ser Leu Lys Asn Glu Thr Asp Ile
             20                  25                  30

Glu Leu Gln Asn Ile Asn His Glu Asp Cys Leu Lys Met Ser Glu Tyr
         35                  40                  45

Glu Asn Val Glu Pro Phe Val Ser Ala Ser Thr Ile Gln Thr Gly Ile
     50                  55                  60

Gly Ile Ala Gly Lys Ile Leu Gly Thr Leu Gly Val Pro Phe Ala Gly
 65                  70                  75                  80

Gln Val Ala Ser Leu Tyr Ser Phe Ile Leu Gly Glu Leu Trp Pro Lys
                 85                  90                  95

Gly Lys Asn Gln Trp Glu Ile Phe Met Glu His Val Glu Glu Ile Ile
            100                 105                 110

Asn Gln Lys Ile Ser Thr Tyr Ala Arg Asn Lys Ala Leu Thr Asp Leu
        115                 120                 125

Lys Gly Leu Gly Asp Ala Leu Ala Val Tyr His Asp Ser Leu Glu Ser
    130                 135                 140

Trp Val Gly Asn Arg Asn Asn Thr Arg Ala Arg Ser Val Val Lys Ser
145                 150                 155                 160

Gln Tyr Ile Ala Leu Glu Leu Met Phe Val Gln Lys Leu Pro Ser Phe
                165                 170                 175

Ala Val Ser Gly Glu Glu Val Pro Leu Leu Pro Ile Tyr Ala Gln Ala
            180                 185                 190

Ala Asn Leu His Leu Leu Leu Leu Arg Asp Ala Ser Ile Phe Gly Lys
        195                 200                 205

Glu Trp Gly Leu Ser Ser Ser Glu Ile Ser Thr Phe Tyr Asn Arg Gln
    210                 215                 220
```

```
Val Glu Arg Ala Gly Asp Tyr Ser Tyr His Cys Val Lys Trp Tyr Ser
225                 230                 235                 240

Thr Gly Leu Asn Asn Leu Arg Gly Thr Asn Ala Glu Ser Trp Val Arg
                245                 250                 255

Tyr Asn Gln Phe Arg Arg Asp Met Thr Leu Met Val Leu Asp Leu Val
            260                 265                 270

Ala Leu Phe Pro Ser Tyr Asp Thr Gln Met Tyr Pro Ile Lys Thr Thr
        275                 280                 285

Ala Gln Leu Thr Arg Glu Val Tyr Thr Asp Ala Ile Gly Thr Val His
    290                 295                 300

Pro His Pro Ser Phe Thr Ser Thr Thr Trp Tyr Asn Asn Asn Ala Pro
305                 310                 315                 320

Ser Phe Ser Ala Ile Glu Ala Ala Val Val Arg Asn Pro His Leu Leu
                325                 330                 335

Asp Phe Leu Glu Gln Val Thr Ile Tyr Ser Leu Leu Ser Arg Trp Ser
            340                 345                 350

Asn Thr Gln Tyr Met Asn Met Trp Gly Gly His Lys Leu Glu Phe Arg
        355                 360                 365

Thr Ile Gly Gly Thr Leu Asn Ile Ser Thr Gln Gly Ser Thr Asn Thr
    370                 375                 380

Ser Ile Asn Pro Val Thr Leu Pro Phe Thr Ser Arg Asp Val Tyr Arg
385                 390                 395                 400

Thr Glu Ser Leu Ala Gly Leu Asn Leu Phe Leu Thr Gln Pro Val Asn
                405                 410                 415

Gly Val Pro Arg Val Asp Phe His Trp Lys Phe Val Thr His Pro Ile
            420                 425                 430

Ala Ser Asp Asn Phe Tyr Tyr Pro Gly Tyr Ala Gly Ile Gly Thr Gln
        435                 440                 445

Leu Gln Asp Ser Glu Asn Glu Leu Pro Pro Glu Ala Thr Gly Gln Pro
    450                 455                 460

Asn Tyr Glu Ser Tyr Ser His Arg Leu Ser His Ile Gly Leu Ile Ser
465                 470                 475                 480
```

```
Ala Ser His Val Lys Ala Leu Val Tyr Ser Trp Thr His Arg Ser Ala
                485                 490                 495

Asp Arg Thr Asn Thr Ile Glu Pro Asn Ser Ile Thr Gln Ile Pro Leu
            500                 505                 510

Val Lys Ala Phe Asn Leu Ser Ser Gly Ala Ala Val Val Arg Gly Pro
        515                 520                 525

Gly Phe Thr Gly Gly Asp Ile Leu Arg Arg Thr Asn Thr Gly Thr Phe
    530                 535                 540

Gly Asp Ile Arg Val Asn Ile Asn Pro Pro Phe Ala Gln Arg Tyr Arg
545                 550                 555                 560

Val Arg Ile Arg Tyr Ala Ser Thr Thr Asp Leu Gln Phe His Thr Ser
                565                 570                 575

Ile Asn Gly Lys Ala Ile Asn Gln Gly Asn Phe Ser Ala Thr Met Asn
            580                 585                 590

Arg Gly Glu Asp Leu Asp Tyr Lys Thr Phe Arg Thr Val Gly Phe Thr
        595                 600                 605

Thr Pro Phe Ser Phe Leu Asp Val Gln Ser Thr Phe Thr Ile Gly Ala
    610                 615                 620

Trp Asn Phe Ser Ser Gly Asn Glu Val Tyr Ile Asp Arg Ile Glu Phe
625                 630                 635                 640

Val Pro Val Glu Val Thr Tyr Glu Ala Glu Tyr Asp Phe Glu Lys Ala
                645                 650                 655

Gln Glu Lys Val Thr Ala Leu Phe Thr Ser Thr Asn Pro Arg Gly Leu
            660                 665                 670

Lys Thr Asp Val Lys Asp Tyr His Ile Asp Gln Val Ser Asn Leu Val
        675                 680                 685

Glu Ser Leu Ser Asp Glu Phe Tyr Leu Asp Glu Lys Arg Glu Leu Phe
    690                 695                 700

Glu Ile Val Lys Tyr Ala Lys Gln Leu His Ile Glu Arg Asn Met
705                 710                 715
```

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<210> 9
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligomer used
      for mutagenesis of the cry1Ba gene

<400> 9
ggacgcatcg tagtgcggac cgtacgaata cgattgg                    37


<210> 10
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: oligomer used
      for mutagenesis of the cry1Ia gene

<400> 10
ggacgcatcg tagtgcggac cgtacaaata caattg                     36
```

Claims

1. *Bacillus thuringiensis* hybrid toxin fragment comprising structural domains I, II and III in this order starting from the N-terminal, wherein the domains are derived from at least two different Cry proteins, domain I is domain I of any *Bacillus thuringiensis* Cry protein or a part of said domain or a peptide substantially similar to said domain, domain II is domain II of Cry1Ia or a part of said domain or a peptide substantially similar to said domain, and domain III is domain III of Cry1Ba or a part of said domain or a peptide substantially similar to said domain.

2. Toxin fragment according to claim 1, wherein domain I is domain I of Cry1Ia or Cry1Ba or a part of said domain or a peptide substantially similar to said domain .

3. Toxin fragment according to claim 1 or 2 comprising an amino acid sequence from about amino acid 20 to about amino acid 641 as shown in SEQ ID NO:2 or an amino acid sequence from about amino acid 20 to about amino acid 632 as shown in SEQ ID NO:4.

4. A hybrid toxin comprising the fragment of any one of the preceding claims , or a toxin at least 85% similar to such a hybrid toxin which has substantially similar insecticidal activity.

5. A toxin according to claim 4 comprising an amino acid sequence as shown in SEQ ID NO:2 or SEQ ID NO:4.

6. Pure proteins which are at least 90% identical to the toxin fragments or hybrid toxins of any of claims 1-5.

7. Recombinant DNA comprising a sequence encoding a protein having an amino acid sequence of the proteins as claimed in any one of claims 1-6.

8. Recombinant DNA according to claim 7 comprising the sequence as shown in SEQ ID N0:1 or 3 or DNA similar

thereto encoding a substantially similar protein.

9. Recombinant DNA according to claim 7 comprising the nucleotide sequence from about nucleotide 170 to about 1929 in SEQ ID N0:1 or from about 147 to about 1896 in SEQ ID NO:3.

10. Recombinant DNA according to any of claims 7-9, which further encodes a protein having herbicide resistance, plant growth-promoting, anti-fungal, anti-bacterial, antiviral and/or anti-nematode properties.

11. Recombinant DNA according to any one of claims 7 to 10 which is modified in that known mRNA instability motifs or polyadenylation signals are removed and/or codons which are preferred by the organism into which the recombinant DNA is to be inserted are used so that expression of the thus modified DNA in the said organism yields substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein components of the hybrid toxin or toxin fragments are endogenous.

12. A DNA sequence which is complementary to one which hybridizes under stringent conditions with the DNA of any one of claims 7 to 11.

13. A vector containing a DNA sequence as claimed in any one of claims 7 to 12.

14. A plant or micro-organism which includes, and enables expression of, the DNA of any one of claims 7-12 or the vector of claim 13.

15. Plants transformed with recombinant DNA as claimed in any one of claims 7 to 12, the progeny of such plants which contain the DNA stably incorporated and hereditable in a Mendelian manner, and/or the seeds of such plants and such progeny.

16. Protein derived from expression of the DNA as claimed in any one of claims 7 to 12 and insecticidal protein produced by expression of the recombinant DNA within plants as claimed in claim 15.

17. An insecticidal composition containing one or more of the proteins as claimed in any one of claims 1-6 and 16.

18. A process for combatting insects which comprises exposing them to proteins or compositions as claimed in any one of claims 1-6, 16 and 17 or the micro-organism of claim 14.

19. An extraction process for obtaining insecticidal proteins, as claimed in any one of claims 1-6 or claim 16, from organic material containing them comprising submitting the material to maceration and solvent extraction, characterized in that the material is a microorganism.

cry1Ba

```
1445
  CTTGGACGCATCGTAGTGCAGATCGTACGAATACGATTGGACC
                   *   *
  5'  GGACGCATCGTAGTGCGGACCGTACGAATACGATTGG   3'
```

cry1Ia

```
1469
  CTTGGACGCATCGTAGTGCAGATCGTACAAATACAATTGAG
                   *   *
  5'  GGACGCATCGTAGTGCGGACCGTACAAATACAATTG   3'
```

FIG. 1

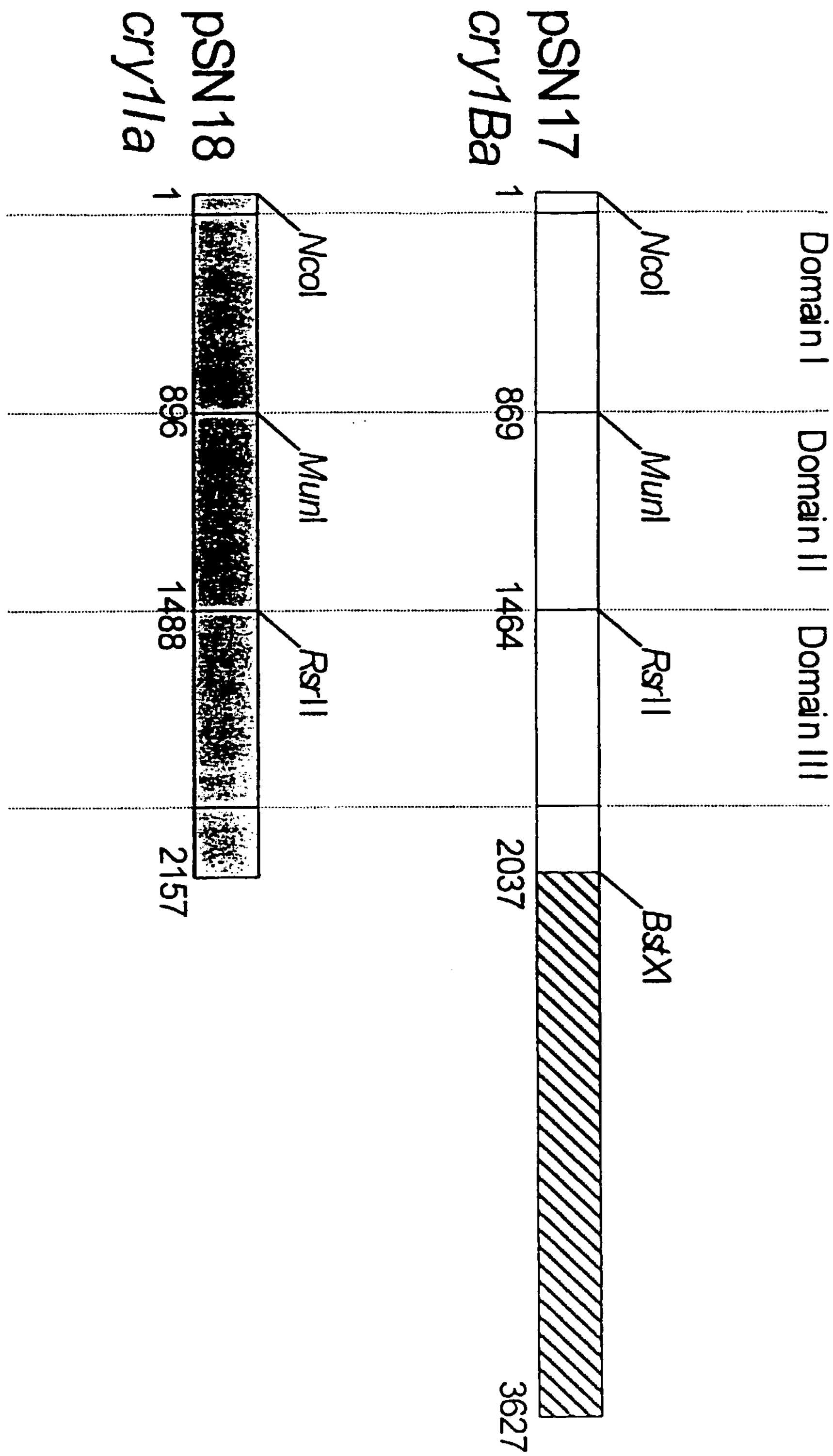
pSN17
cry1Ba
pSN18
cry1Ia
Domain I
Domain II
Domain III
NcoI
MunI
RsrII
BstXI
1
869
1464
2037
3627
1
896
1488
2157

FIG. 2

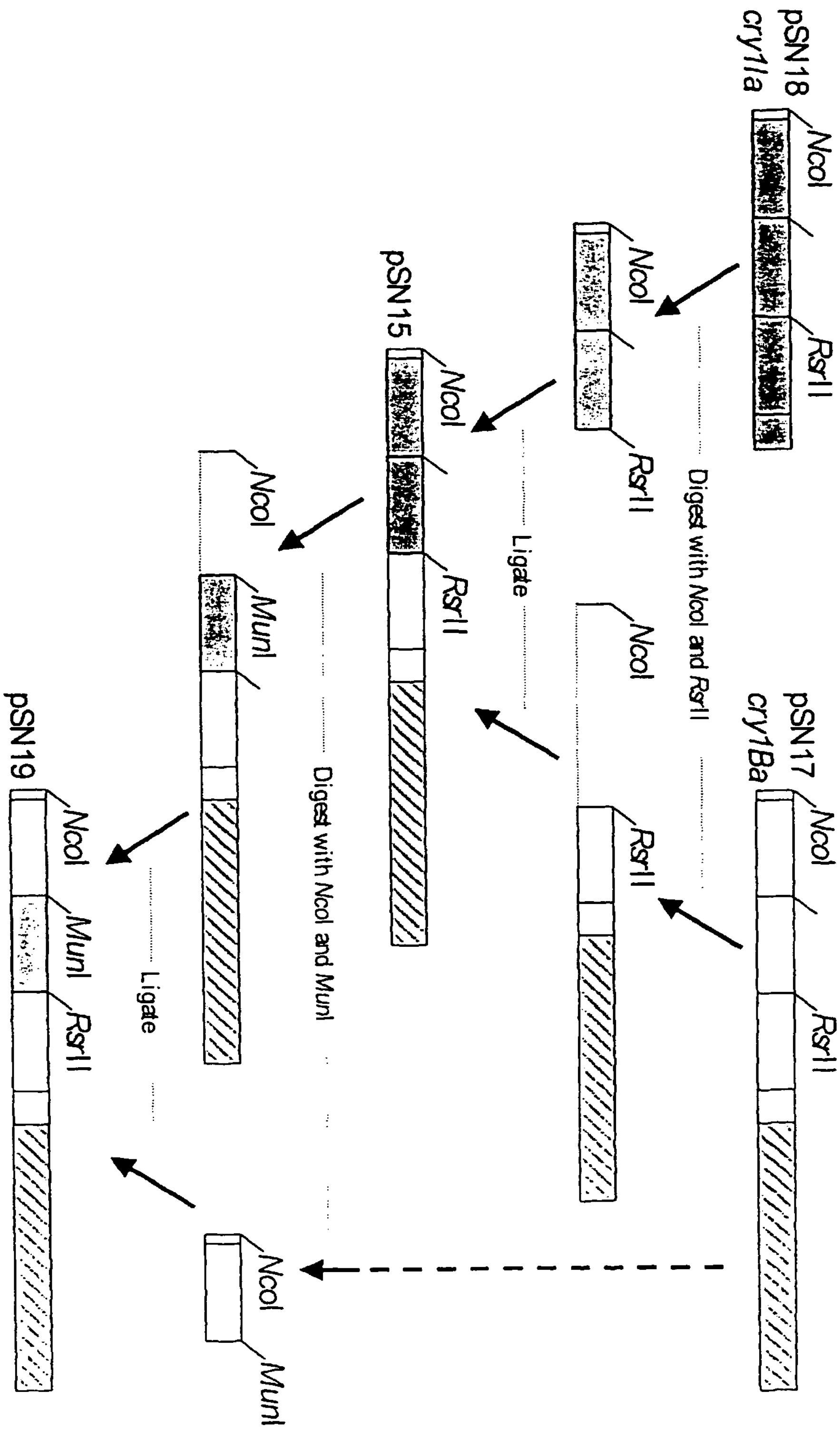
pSN18
cry1Ia
NcoI
RsrII
NcoI
RsrII
Digest with NcoI and RsrII
pSN17
cry1Ba
NcoI
RsrII
NcoI
RsrII
Ligate
pSN15
NcoI
RsrII
NcoI
MunI
Digest with NcoI and MunI
NcoI
MunI
Ligate
pSN19
NcoI
MunI
RsrII

FIG. 3

A

```
                      RsrII
cry1Ia 1471TGGACGCATCGTAGTGCG........a.....a...
cry1Ba 1447..................GACCGTACGAATACGATT
           TrpThrHisArgSerAlaAspArgThrAsnThrIle
```

B

```
                        MunI
cry1Ba  853GAAGTTTATACAGACGC......ag..ac.ggggta
cry1Ia  880.....a...........AATTGGGACAGTACATCCG
           GluValTyrThrAspAlaIleGlyThrValHisPro
```

FIG. 4

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 20 3723

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 99 50293 A (NOVARTIS ERFINDUNGEN VERWALTUN ;NOVARTIS AG (CH); DESAI NALINI MAN) 7 October 1999 (1999-10-07) * page 4 - page 7 * * claims; examples * ---	4,6,7, 11-18	C12N15/32 C12N15/62 C07K14/325 A01H5/00 A01N63/00
D,X	WO 95 06730 A (SANDOZ LTD ;SANDOZ AG (DE); SANDOZ AG (AT); BOSCH HENDRIK JAN (NL)) 9 March 1995 (1995-03-09)	10,19	
A	* the whole document * ---	1-18	
A	VISSER ET AL: "Domain-function studies of Bacillus thuringiensis crystal proteins: a genetic approach" ENTWISTLE, P.F. (EDS) : "BACILLUS THURINGIENSIS, AN ENVIRONMENTAL BIOPESTICIDE: THEORY AND PRACTICE" ,1993, pages 71-88, XP002086356 GB,CHICHESTER, WILEY & SONS * page 80 - page 84 * ---	1-18	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	ELY S: "THE ENGINEERING OF PLANTS TO EXPRESS BACILLUS THURINGIENSIS DELTA-ENDOTOXINS" ENTWISTLE, P.F. (EDS) : "BACILLUS THURINGIENSIS, AN ENVIRONMENTAL BIOPESTICIDE: THEORY AND PRACTICE" ,1993, pages 105-124, XP002054693 GB,CHICHESTER, WILEY & SONS * page 115 - page 120 * --- -/--	11-16	C07K A01N C12R

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 March 2000	Andres, S

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

Application Number
EP 99 20 3723

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- [ ] Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

- [ ] No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

- [ ] All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

- [x] As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

- [ ] Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

- [ ] None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 20 3723

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,A	BRADLEY D ET AL: "The Insecticidal CryIB Crystal Protein of Bacillus thuringiensis ssp. thuringiensis Has Dual Specificity to Coleopteran and Lepidopteran Larvae." JOURNAL OF INVERTEBRATE PATHOLOGY , vol. 65, no. 2, 1995, pages 162-173, XP000891272 ISSN: 0022-2011 ---		
D,A	MAAGD DE R A: "DIFFERENT DOMAINS OF BACILLUS THURINGIENSIS DELTA-ENDOTOXINS CAN BIND TO INSECT MIDGUT MEMBRANE PROTEINS ON LIGAND BLOTS" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 8, August 1996 (1996-08), pages 2753-2757, XP002061801 ISSN: 0099-2240 -----		
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 March 2000	Andres, S

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

LACK OF UNITY OF INVENTION SHEET B

Application Number
EP 99 20 3723

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-18

   Hybrid Bacillus thuringiensis insecticidal toxins comprising a domain II derived from Cry1Ia and a domain III derived from Cry1Ba. Nucleic acids encoding the toxins, vectors containing them, plants transformed therewith, and compositions containing the proteins.

2. Claim : 19

   An extraction process for obtaining insecticidal proteins.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 99 20 3723

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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20-03-2000

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		ZA 9406770 A	04-03-1996

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82